Method for the recombinant expression of an N-terminal fragment of hepatocyte growth factor

The invention relates to a method for the recombinant expression of a N-terminal four kringle-containing fragment of hepatocyte growth factor.

Background of the Invention

Hepatocyte growth factor (HGF/SF) is a polypeptide identified and purified by Nakamura, T., et al., Biochem. Biophys. Res. Commun. 22 (1984) 1450-1459. It was further found that hepatocyte growth factor is identical to scatter factor (SF), Weidner, K.M., et al., Proc. Natl. Acad. Sci. USA 88 (1991) 7001-7005. HGF is a glycoprotein involved in the development of a number of cellular phenotypes including proliferation, mitogenesis, formation of branching tubules and, in the case of tumor cells, invasion and metastasis. For a status review, see Stuart, K.A., et al., Int. J. Exp. Pathol. 81 (2000) 17-30.

Both rat HGF and human HGF have been sequenced and cloned (Miyazawa, K. et al., Biochem. Biophys. Res. Comm. 163 (1989) 967-973; Nakamura, T., et al., Nature 342 (1989) 440-443; Seki, T., et al., Biochem. and Biophys. Res. Comm. 172 (1990) 321-327; Tashiro, K., et al., Proc. Natl. Acad. Sci. USA 87 (1990) 3200-3204; Okajima, A., et al., Eur. J. Biochem. 193 (1990) 375-381).

HGF is a protein with high similarity to human plasminogen (38% amino acid sequence identity). HGF and plasminogen are both synthesized as a single chain polypeptide which is proteolytically processed to a disulfide-linked heterodimer. HGF contains an N-terminal domain four consecutive kringle domains and a carboxyterminal protease-like domain. Different truncated HGF variants have been described. NK1 is the shortest HGF variant described. NK1 contains amino acids 32-210 and is truncated after the first kringle domain (Lokker, N.A., and Godowski, P.J., J. Biol. Chem. 268 (1993) 17145-17150). NK2 consists of the N-terminal amino acid terminus and kringle 1 and kringle 2 and is the naturally occurring product of an alternatively spliced HGF mRNA (Chan, A.M., et al., Science 254 (1991) 1382-1385). Further HGF variants containing parts of the heavy chain of HGF (amino acids 1-494, containing the alpha-subunit of HGF from amino acids 1-463) are described by Lokker, N.A., EMBO J. 11 (1992) 2503-2510).

It was further found that an HGF/SF fragment, termed NK4, consisting of the N-terminal hairpin domain and the four kringle domains of HGF/SF has pharmacological properties that are completely different from those of HGF/SF, and is an antagonist to the influence of HGF/SF on the motility and the invasion of colon cancer cells, and is, in addition, an angiogenesis inhibitor that suppresses tumor growth and metastasis (Parr, C., et al., Int. J. Cancer 85 (2000) 563-570; Kuba, K., et al., Cancer Res. 60 (2000) 6737-6743; Date, K., et al., FEBS Lett. 420 (1997) 1-6; Date, K., et al., Oncogene 17 (1989) 3045-3054).

NK4 is prepared according to the state of the art (Date, K., et al., FEBS Lett. 420 (1997) 1-6) by recombinant expression of HGF cDNA in CHO cells and subsequent digestion with pancreatic elastase. Two other isoforms of HGF (NK1 and NK2) encoding the N-terminal domain and kringle 1, and the N-terminal domain and kringles 1 and 2, respectively, were produced in E.coli (Stahl, S.J., Biochem. J. 326 (1997) 763-772). However, this method results only in about an amount of HGF-derived proteins which is about 10-20% of the total protein.

Summary of the Invention

The invention provides a method for the production of the alpha-chain of HGF or a fragment thereof (NK polypeptide) by expression of a nucleic acid encoding said NK polypeptide in a microbial host cell, isolation of inclusion bodies containing said NK polypeptide in denatured form, solubilization of the inclusion bodies and naturation of the denatured NK polypeptide, characterized in that in said nucleic acid at least one of the codons of amino acids selected from the group consisting of codons at positions 33, 35 and 36 is CGT.

Amino acid (aa) and codon numbering is according to the sequence shown in Swiss-Prot P14210, wherein aa (amino acid) 1-31 denotes signal sequence, aa 32-494 denotes alpha chain, aa 128-206 kringle 1, aa 211-288 kringle 2, aa 305-383 krinlge 3 and aa391-469 kringle 4.

Surprisingly it was found, that modification of at least one of the codons of the DNA sequence of positions 33, 35 and 36 (codon 33, 35 and 36 encode arginine, numbering according to M73239) results in an increase of the expression yield of about 100% or more. It is further preferred that the codon for amino acid 32 is

changed from encoding Gln to encoding Ser in order to improve splitting off N-terminal methionine.

NK polypeptides according to the invention consist of aa 32-494 or a N-terminal fragment thereof (always beginning with aa32), preferably fragment aa 32-478, the smallest fragment being aa 32-207. All NK polypeptides according to the invention show activity in a scatter assay according to Example 4.

The invention further provides a nucleic acid encoding an NK polypeptide consisting of aa 32-494 or an N-terminal fragment thereof, beginning with aa 32, preferably fragments aa 32-x, wherein x is a number between 207 and 478, and x is preferably 207 or 478, characterized in that at least one of the codons of amino acids selected from the group consisting of codons at positions 33, 35 and 36 is CGT. Preferably, all codons at positions 33, 35 and 36 are CGT.

In a preferred embodiment of the invention aa 32 is changed from glutamine to serine to improve homogeneity of the protein (cleavage of N-terminal methionine).

It is further preferred to introduce two translational stop codons (TAA, TAG and/or and TGA) at the end of the nucleic acid encoding the NK polypeptide in order to stop the translation at a position equivalent to the end of desired polypeptide.

Detailed Description of the Invention

Human HGF is a disulfide-linked heterodimer, which can be cleaved in an α-subunit of 463 amino acids and a β-subunit of 234 amino acids, by cleavage between amino acids R494 and V495. The N-terminus of the α-chain is preceded by 31 amino acids started with a methionine group. This segment includes a signal sequence of 31 amino acids. The α-chain starts at amino acid 32 and contains four kringle domains. The so-called "hairpin domain" consists of amino acids 70-96. The kringle 1 domain consists of amino acids 128-206. The kringle 2 domain consists of amino acids 211-288, the kringle 3 domain consists of amino acids 305-383, and the kringle 4 domain consists of amino acids 391-469 of the α-chain, approximately. There exist variations of these sequences, essentially not affecting the biological

properties of NK polypeptides (especially not affecting its activities antagonistic to HGF and its antiangiogenic activities), which variations are described, for example, in WO 93/23541. Also the length of NK polypeptides can vary within a few amino acids as long as its biological properties are not affected.

NK1 consists of aa 32 to 206-210 of the HGF/SFα-chain, NK2 consists of aa32 to 288-305 and NK4 is composed of aa 32 to 447 (resp.469-494). Further NK polypeptides encoded by the nucleic acids according to the invention and which can be produced recombinantly according to the invention are described in WO 93/23541 and are e.g. 32-207, 32-303, or 32-384. NK polypeptides have the in vivo biological activity of causing inhibition of tumor growth, angiogenesis and/or metastasis.

The NK polypeptides can be produced by recombinant means in prokaryotes. For expression in prokaryotic host cells, the nucleic acid is integrated into a suitable expression vector, according to methods familiar to a person skilled in the art. Such an expression vector preferably contains a regulatable/inducible promoter. The recombinant vector is then introduced for the expression into a suitable host cell such as, e.g., E. coli and the transformed cell is cultured under conditions which allow expression of the heterologous gene. After fermentation inclusion bodies containing denatured NK polypeptide are isolated.

Escherichia, Salmonella, Streptomyces or Bacillus are for example suitable as prokaryotic host organisms. For the production of NK polypeptides prokaryotes are transformed in the usual manner with the vector which contains the DNA according to the invention and encoding a NK polypeptide and subsequently fermented in the usual manner. However expression yield in E. coli using the original DNA sequence of a NK polypeptide (GenBank M73239) is very low.

Inclusion bodies are found in the cytoplasm as the gene to be expressed does not contain a signal sequence. These inclusion bodies are separated from other cell components, for example by centrifugation after cell lysis.

The inclusion bodies were solubilized by adding a denaturing agent like 6 M guanidinium hydrochloride or 8 M urea at pH 7-9 in phosphate buffer (preferably in a concentration of 0.1 – 1.0 M, e.g.0.4 M) preferably in the presence of DTT

(Dithio-1,4-threitol). The solubilisate is diluted in phosphate buffer pH 7-9 in the presence of GSH/GSSG (preferably 2-20 mM, glutahtion) and a denaturing agent in a non denaturing concentration (e.g. 2M guanidinium hydrochloride or 4 M urea) or preferably instead of guanidinium hydrochloride or urea, arginine in a concentration of about 0.3 to 1.0 M, preferably in a concentration of about 0.7M. Renaturation is performed preferably at a temperature of about 4 C and for about 48 to160 hours.

According to the state of the art the use of Tris buffer during solubilization and naturation leads to a considerable amount (of about 50%) of side-products which are identified by the inventors as consisting mainly of GSH-modified NK polypeptides. To the contrary, it was surprisingly found that the use of potassium phosphate buffer in a pH range between 7 and 9, preferably between pH 8 and 9, leads to a considerable improvement in yield and purity of NK polypeptides.

After naturation is terminated the solution was dialyzed preferably against phosphate buffer pH 7-9 (preferably in a concentration of 0.1 – 1.0 M, e.g. 0.3 M) for at least 24 hours, preferably for 24 – 120 hours.

NK polypeptides can be purified after recombinant production and naturation of the water insoluble denatured polypeptide (inclusion bodies) according to the method of the invention preferably by chromatographic methods, e.g. by affinity chromatography, hydrophobic interaction chromatography, immunoprecipitation, gel filtration, ion exchange chromatography, chromatofocussing, isoelectric focussing, selective precipitation, electrophoresis, or the like. It is preferred to purify NK polypeptides by hydrophobic interaction chromatography, preferably at pH 7-9, in the presence of phosphate buffer and/or by the use of butyl- or phenyl sepharose.

The following examples, references, figure and sequence listing are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

Description of the Figure:

Figure 1: SDS-Gel (10% NuPAGE-SDS, 5µl per lane, numbering from left to right) of NK4 protein in biomass and isolated inclusion bodies (IB).

lane 1: standard
lane 2: biomass
lane 3: supernant after centrifugation
lane 4: supernant after further centrifugation
lane 5: IB preparation
lane 6: IB preparation after wash

Description of the Sequences:

SEQ ID NO:1 Amino acid sequence and DNA sequence encoding the α-chain of HGF, original sequence according to GenBank M73239 (without signal sequence)

SEQ ID NO:2 Protein sequence of the α-chain of HGF

SEQ ID NO:3 Amino acid sequence and DNA sequence encoding NK4 according to the invention (amino acid sequence including N-terminal methionine, DNA sequence including two stop codons)

SEQ ID NO:4 Protein sequence of NK4

Example 1

Recombinant expression of NK polypeptides

The NK4 polypeptide consisting of amino acid position 32 to 478 of HGF was used for cloning and recombinant expression in Escherichia coli. The original DNA sequence used as source of DNA was described (database identifier "gb:M73239"). PCR was performed in order to amplify and concurrently modify the DNA coding for NK4 (SEQ ID NO: 1). All methods were performed under standard conditions.

In comparison to the original DNA sequence of NK4, the following modifications were introduced:

- Elimination of the eukaryotic signal peptide sequence and fusion of the ATG start codon next to amino acid position 32 of NK4
- exchange of amino acid position 32 (position 2 in SEQ ID NO:2) from Gln to Ser in order to improve homogeneity of the protein product (Met-free)
- modification of the DNA sequence of the codons of amino acids at position 33 (AGG to CGT), 35 (AGA to CGT), and 36 (AGA to CGT) in order to improve gene expression in E.coli.
- modification of the DNA sequence of codons at position 477 (ATA to ATC) and 478 (GTC to GTT) in order to facilitate insertion of PCR product into the vector
- introduction of two translational stop codons at positions 479 (TAA) and 480 (TAG), in order to stop the translation at a position equivalent to the end of NK4 protein domain.

The PCR-amplified DNA fragment was treated with restriction endonucleases NdeI and BanII and was ligated to the modified pQE vector (Qiagen) (elimination of His-tag as well as DHFR coding region), which was appropriately treated with NdeI and BanII. The elements of expression plasmid pQE-NK4-Ser (plasmid size 4447 bp) are T5 promotor/lac operator element, NK4 coding region, lambda to transcriptional termination region, *rrn*B T1 transcriptional termination region, ColE1 origin of replication and β-lactamase coding sequence.

The ligation reaction was used to transform E.coli competent cells, e.g. E. coli strain C600 harbouring expression helper plasmid pUBS520 (EP 0 373 365). E.coli colonies were isolated and were characterized with respect to restriction and sequence analysis of their plamsids. The selection of clones was done by analysis of the NK4 protein content after cultivation of recombinant cells in LB medium in the presence of appropriate antibiotics and after induction of the gene expression by addition of IPTG (1mM). The protein pattern of cell lysates were compared by PAGE. The recombinant E.coli clone showing the highest proportion of NK4 protein was selected for the production process. Fermentation was performed under standard conditions and inclusion bodies were isolated. Yield: 130 g/l net weight of cells with 30%-40% NK4 of total protein.

NK1 and NK2 can be produced recombinantly in an analogous manner.

Example 2

Solubilization and naturation

Inclusion bodies were dissolved over night in a buffer containing 6 M guanidinium hydrochloride, 0.1 M potassium phosphate pH 8.5 (by titration with 10 M KOH), 1 mM EDTA, 0.01 mM DTT. The concentration of the dissolved protein was determined by Biuret assay and finally adjusted to a concentration of 25 mg total protein/ml at room temperature.

This NK-solubilisate was diluted to a concentration of 0.4 mg/ml in a buffer containing 0.7 M arginine, 0.1 M potassium phosphate pH 8.5 (by titration with conc. HCl), 10 mM GSH, 5 mM GSSG and 1 mM EDTA. This renaturation assay was incubated between 2 and 8 days at 4°C. After obtaining the maximal renaturation efficacy, the renaturation assay of 15 l volume was concentrated to 3 l using a tangential flow filtration unit (MW cut off: 10 kDa, Sartorius). It was subsequently dialyzed against 3 times 50 l buffer containing 0.3 M potassium phosphate at pH 8.0 for at least 3 x 24 hours, optimally for 5 days in total.

Example 3

Purification

Purification was performed by Heparin-Sepharose chromatography.

Buffer conditions:
Buffer A: 50 mM Tris pH 8.0
Buffer B: 50 mM Tris pH 8.0, 2 M NaCl

Gradient:	5-25%	buffer B, 2 column volumes
	25-55%	buffer B, 16 column volumes
	55-100%	buffer B, 0.7 column volumes
	100%	buffer B, 2 column volumes

To the eluted material 1 M ammonium sulfate in 0.1 M potassium phosphate pH 8.0 was added and incubated at 4°C overnight. The sample was centrifuged and the

supernatant was loaded on a Phenyl Sepharose column (150 ml). The column was washed with 1 column volume 1 M ammonium sulfate, 50 mM potassium phosphate pH 8.0.

Elution conditions:
Buffer A: 1 M ammonium sulfate, 50 mM potassium phosphate pH 8.0
Buffer B: 50 mM potassium phosphate pH 8.0, 40 % ethylene glycol
0-100 % buffer B, 20 column volumes

Example 4

Determination of activity

a) **Scatter assay**

MDCK cells were subconfluently grown in tissue culture plates. Cells were treated with HGF (10 ng/ml) or with combinations of HGF and NK4. In these experiments the HGF-induced cell scattering was inhibited by the addition of a 10 to 1000-fold molar excess of NK4 at least for 90% and more, showing the functional activity.

b) **Proliferation assay**

Inhibition of the mitogenic activity of HGF by NK4 was determined by measuring DNA synthesis of adult rat hepatocytes in primary culture as described in Nakamura et al. (1989). In these experiments the HGF-induced cell proliferation was inhibited by the addition of a 10 to 1000-fold molar excess of NK4 at least for 90% and more, showing the functional activity.

c) **Invasion assay**

In this assay the invasive potential of tumor cells is analyzed. The assay was done essentially as decribed in Albini, A., et al., Cancer Res. 47 (1987) 3239-3245, using HT115 cells. Again, HGF-induced (10 ng/ml) cell invasion could be inhibited by a 10 to1000-fold molar excess of NK4 at least for 90% and more, showing the functional activity.

Example 5

Activity in vivo

Model:	Lewis Lung Carcinoma nude mouse tumor model 1 x 10^6 Lewis Lung Carcinoma cells were s.c. implanted into male nude mice (BALB/c nu/nu).
Treatment:	After 4 days, one application daily of pegylated NK4 over a period of 2-4 weeks
Dose:	1000 µg/mouse/day 300 µg/mouse/day 100 µg/mouse/day placebo
Result:	Treatment with NK4 shows a dose dependent suppression of primary tumor growth and metastasis, whereas no effect is seen in placebo treated groups.

List of References

Albini, A., et al., Cancer Res. 47 (1987) 3239-3245

Chan, A.M., et al., Science 254 (1991) 1382-1385

Date, K., et al., FEBS Lett. 420 (1997) 1-6

Date, K., et al., Oncogene 17 (1989) 3045-3054

EP 0 373 365

Kuba, K., et al., Cancer Res. 60 (2000) 6737-6743

Lokker, N.A., and Godowski, P.J., J. Biol. Chem. 268 (1993) 17145-17150

Lokker, N.A., EMBO J. 11 (1992) 2503-2510

Miyazawa, K. et al., Biochem. Biophys. Res. Comm. 163 (1989) 967-973

Nakamura, T., et al., Biochem. Biophys. Res. Commun. 22 (1984) 1450-1459

Nakamura, T., et al., Nature 342 (1989) 440-443

Okajima, A., et al., Eur. J. Biochem. 193 (1990) 375-381

Parr, C., et al., Int. J. Cancer 85 (2000) 563-570

Seki, T., et al., Biochem. and Biophys. Res. Comm. 172 (1990) 321-327

Stahl, S.J., Biochem. J. 326 (1997) 763-772

Stuart, K.A., et al., Int. J. Exp. Pathol. 81 (2000) 17-30

Tashiro, K., et al., Proc. Natl. Acad. Sci. USA 87 (1990) 3200-3204

Weidner, K.M., et al., Proc. Natl. Acad. Sci. USA 88 (1991) 7001-7005

WO 93/23541